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Centrally acting adrenomedullin in the long-term potentiation of sympathetic vasoconstrictor activity induced by intermittent hypoxia in rats

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New findings

What is the central question of this study?

Adrenomedullin in the rostral ventrolateral medulla (RVLM) increases sympathetic activity. Given that adrenomedullin is released during hypoxia, we explored its antagonism and agonism in the RVLM after chronic intermittent hypoxia (CIH) exposure.

What is the main finding and its importance?

We found that CIH exposure sensitizes adrenomedullin-dependent mechanisms in the RVLM, supporting its role as a sympathoexcitatory neuromodulator. Our data identify a novel mechanism for the generation of sympathetic overdrive and hypertension associated with hypoxia, providing a potential guidance on new therapeutic approaches for controlling sympathetic hyperactivity in diseases such as sleep apnoea and neurogenic hypertension.

Abstract

Adrenomedullin in the rostral ventrolateral medulla (RVLM) has been shown to increase sympathetic activity whereas the antagonism of its receptors inhibited this autonomic activity lowering blood pressure in conditions of hypertension. Given that hypoxia is a stimulant for releasing adrenomedullin, we hypothesized that the presence of this peptide in the RVLM associated with chronic intermittent hypoxia (CIH) would cause sympathetic overdrive. Juvenile male rats (50-55 g) submitted to CIH (6% oxygen every 9 min, 8 h/day for 10 days) were studied in an arterially perfused *in situ* preparation where sympathetic activity was recorded. In control rats (n=6), exogenously applied adrenomedullin in the RVLM raised baseline sympathetic activity when combined with episodic activation of peripheral chemoreceptors (KCN 0.05%, 5 times every 5 min). This sympathoexcitatory response was markedly amplified in rats previously exposed to CIH (n=6). The antagonism of adrenomedullin receptors in the RVLM caused a significant reduction in sympathetic activity in CIH group (n=7), but not in controls (n=8). The transient reflex evoked sympathoexcitatory response to peripheral chemoreceptor stimulation was not affected by either adrenomedullin or adrenomedullin receptor antagonism in the RVLM of control and CIH rats. Our findings indicate that CIH sensitizes the sympathoexcitatory networks within the RVLM to adrenomedullin, supporting its role as an excitatory neuromodulator when intermittent hypoxia is present. These data reveal novel state-dependent mechanistic insights into the generation of sympathetic overdrive and provide potential guidance on possible unique approaches for controlling sympathetic discharge in diseases such as sleep apnoea and neurogenic hypertension.

INTRODUCTION

Persistent sympathetic overactivity is an established common feature of numerous diseases (Julius & Nesbitt, 1996; Canale et al., 2013) yet its aetiology remains enigmatic. Unequivocally, understanding the mechanisms involved in both raising and sustaining levels of sympathetic nerve activity (SNA) remains crucial for designing novel therapeutic strategies and addressing unmet clinical need. One such need is sleep apnoea. Sleep apnoea prevalence is up to 15% in middle-aged adults and is characterized by repetitive bouts of oxygen desaturations (apnoea-hypopnoea index ≥ 15) that triggers hypertension caused mainly through persistent over activation of the sympathetic nervous system (Simpson et al., 2013; Arnardottir et al., 2016; McNicholas, 2017). Because patients remain hypertensive during the day, the chronic intermittent hypoxia (CIH) that occurs at night induces plasticity within neural circuits controlling sympathetic activity. Understanding what these sensitisation mechanisms are that produce long-term facilitation of sympathetic activity is clearly important for controlling sympathetic activity and hypertension in sleep apnoea but may have wider implications for all diseases where sympathetic overdrive is rife. However, given that continuous positive airway pressure requires >5.5 hours per night to reduce sympathetic activity and blood pressure and as a result is poorly tolerated (Fava *et al.*, 2014; Furlan *et al.*, 2015) this reinforces the need to understand sympathetic activity generation during CIH. This challenge was the main aim of this study.

We have considered the peptide adrenomedullin acting on the sympathoexcitatory neural networks of the rostral ventrolateral medulla (RVLM) – a major region for generation of sympathetic activity destined for the cardiovascular system (Guertzenstein & Silver, 1974; Ross *et al.*, 1984b; Sun *et al.*, 1988). Adrenomedullin is a 52-amino acid peptide, initially isolated from human pheochromocytoma and is known to be a potent peripheral vasorelaxant peptide (Kitamura *et al.*, 1993; Nuki *et al.*, 1993). It is synthesised and secreted from both vascular smooth muscle and endothelial cells (Minamino *et al.*, 2002). Intriguingly, the International Consortium for Blood Pressure Genome-Wide Association *et al.* (2011) identified from a genome wide association study that adrenomedullin and one of its receptors were variants in a meta-analysis of blood pressure whereas Verweij *et al.* (2013) reported genetic variants in the genes encoding pre-pro-adrenomedullin. These variants were interpreted to functionally affect the vascular system. However, adrenomedullin has also been found within the brain (Sakata *et al.*, 1994; Ueta *et al.*, 2001; Serrano *et al.*, 2002b) and in contrast to its function in the periphery of lowering blood pressure (Ishiyama *et al.*, 1993;

Kitamura *et al.*, 1993; Nuki *et al.*, 1993), it caused hypertension (Saita *et al.*, 1998; Shan & Krukoff, 2001b; Xu & Krukoff, 2004) via excitation of pre-sympathetic pre-motor neurones located in the RVLM (Fan *et al.*, 2006). Most relevant to this study is that adrenomedullin synthesis and release is known to be triggered by ischemia and hypoxia in both the periphery (Minamino *et al.*, 2002; Belloni *et al.*, 2004) and within the brain (Serrano *et al.*, 2002a). Given that both acute (Dick *et al.*, 2007; Xing & Pilowsky, 2010; Lemes *et al.*, 2016) and chronic intermittent hypoxia (Fletcher *et al.*, 1992; Zoccal *et al.*, 2008; Zoccal *et al.*, 2009a) cause long-term facilitation of sympathetic activity and hypertension, we tested the hypothesis that combining exposure of rats to CIH with adrenomedullin in the RVLM would sensitize sympathoexcitatory neural networks leading to persistent sympathetic overdrive.

MATERIALS AND METHODS

Ethical Approval

All procedures were carried out according to the UK Animals (Scientific Procedures) Act 1986, under licence to the Home Office and were approved by the University of Bristol, the University of São Paulo and the São Paulo State University animal welfare committees (Protocols 019/2006 and 18/2014). Methods and experiments also conform to the principles and regulations of journal policy and regulations on animal experimentation (Grundy, 2015). Experiments were conducted on weaned male Wistar rats (50-55 g) bred within the University of São Paulo and the São Paulo State University's animal facilities and housed with controlled temperature (23 ± 2 ºC) with humidity control (55 ± 10% v/v), and a 12/12h light/dark period (lights on at 07:00 h) and ad libitum access to food and water.

Animal studies

The rats were exposed to CIH as previously described (Zoccal *et al.*, 2008). Briefly, the animals were housed in collective cages (maximum of 5 animals per cage) and maintained inside chambers equipped with gas injectors as well as sensors of O_2, CO_2, humidity and temperature, at controlled conditions of temperature (22±1 °C) and humidity (55±10 % v/v). The CIH protocol consisted of 5 minutes of normoxia (FiO_2 of 20.8% v/v) followed by 4 minutes of pure N_2 injection into the chamber to reduce the FiO_2 to 6.2-6% (v/v), remaining at this level for 40 seconds. After this hypoxic period, pure O_2 was injected to return the FiO_2 back to 20.8% (v/v). This 9-minute cycle was repeated 8 hours a day (from 9:30 am to 5:30 pm) for 10 days. During the remaining 16 hours, the animals were maintained at a FiO_2 of 20.8% (v/v). The injections of N_2 and O_2 (White Martins, São Carlos,

Brazil) were regulated by a solenoid valve system controlled by a computerized system (Oxycycler, Biospherix, USA). In an identical chamber in the same room, the control rat group was exposed to a FiO_2 of 20.8% (v/v) 24 hours a day for 10 days. The control rats were also exposed to a similar valve noise due to the frequent injection of O_2 to maintain the FiO_2 at 20.8% (v/v). In both CIH and control chambers, the gas injections were performed at the upper level of the chamber in order to avoid direct jets of gas impacting on the animals, which could cause stress.

Arterially perfused in situ juvenile rat preparation

After CIH or normoxia exposure, rats were surgically prepared as originally described (Paton, 1996) to obtain the *in situ* preparations. In brief, rats were anesthetized using isoflurane (5% v/v). A deep level of anaesthesia was assessed by a failure to respond to a noxious pinch of either a paw or the tail and an absence of a corneal reflex. The rats were then transected below the diaphragm, exsanguinated (which resulted in euthanasia) and their upper body submerged in ice-cooled Ringer solution. The cranium was opened and rats decerebrated at the pre-collicular level. The preparation was skinned, transferred to a recording chamber and a double-lumen catheter was inserted into the descending aorta. One lumen was used to deliver perfusate pumped using a roller pump (Watson Marlow 505S). The perfusate was an isosmotic Ringer solution containing in mM: NaCl 120, $NaHCO_3$ 24, KCl 3, $CaCl_2$ 2.5, $MgSO_4$ 1.25, KH_2PO_4 1.25, glucose 10, plus an oncotic agent (polyethylene glycol, 1.5% w/v; Sigma-UK), gassed with carbogen (95% v/v O_2 and 5% CO_2 $_{v/v}$), warmed to 32°C, pH 7.3 after carbogenation, and filtered using a nylon screen (pore size: 25 μm). The second lumen of the catheter was used to monitor aortic perfusion pressure, which was maintained in the range of 60-70 mmHg by adjusting the rate flow to 21-25 $mL.min^{-1}$ and by adding vasopressin to the perfusate (0.6 - 1.2 nM, Sigma, St. Louis, MO, USA). When necessary, additional increments in the flow rate or AVP in the perfusate were applied (10-20 % of the initial values) in some preparations after 60-90 min to maintain in the perfusion pressure levels throughout experiments and to avoid any pressure-related changes. The head of the preparation was fixed by ear bars and a nasal clamp so that the dorsal surface of the brainstem was positioned horizontally. The cerebellum was removed to enhance access to the dorsal brainstem for intraparenchymal microinjections. The left phrenic nerve (PN) was isolated and its activity recorded from the cut central end using a glass suction bipolar electrode held in a 3-D micromanipulator. Rhythmic, ramping PN activity gave a continuous

physiological index of preparation viability. After respiratory-related movements re-commenced, a neuromuscular blocker (vecuronium bromide, 40 μg ml^{-1}, Cristália, Itapira, BR) was added to the perfusate to mechanically stabilize the preparation. Sympathetic nerve activity was recorded from the thoracic sympathetic chain (tSN) using a bipolar glass suction electrode. All nerve signals were AC-amplified (P511, Grass Technologies, Middleton, USA), band-pass filtered (0.1−3 kHz), rectified and displayed as a moving average using a 50 ms time constant. At the end of each experiment the background electrical noise was measured after application of lidocaine (2%) to the sympathetic chain; this level was subtracted from the integrated signal.

Microinjections into the RVLM

Using glass micropipettes (20-30 μm tip diameter), microinjections of adrenomedullin (100 fmol/100 nl, American Peptides, Sunnyvale, USA) or AM 22-52 (adrenomedullin type 1 and 2 receptor antagonist, 100 pmol/100 nl, American Peptides) were made bilaterally in the RVLM using the following coordinates: 400-500 μm rostral to the rostral pole of area postrema, 1600-1700 μm lateral to the midline and 3.8-4.0 mm ventral to the dorsal surface of the brainstem. The concentrations of agonist and antagonist were determined based on previous studies demonstrating the effects of adrenomedullin in the RVLM of anaesthetised rats (Xu & Krukoff, 2004). The volume microinjected was determined by viewing the movement of the meniscus through a binocular microscope fitted with a pre-calibrated eyepiece reticule. The RVLM microinjection sites were identified physiologically by microinjection of L-glutamate (1 nmol/100 nl) to evoke an increase in SN activity (Moraes *et al.*, 2012). Vehicle controls were performed using saline (100 nl). In some experiments, fluorescent latex microbeads were added to the glutamate solution to also allow histological verification of the injection sites (see below).

Experimental protocol

Initially baseline PN and tSN activities were recorded for 20-30 min and then peripheral arterial chemoreceptors were stimulated using potassium cyanide (KCN, 0.05% w/v, 50 μl; injected into a side port of the arterial perfusion catheter). We *a priori* determined that this dose was sub-maximal and supra-threshold. After the RVLM was functionally identified (increases in tSN to glutamate), bilateral microinjections of adrenomedullin were performed into the RVLM of both control and CIH treated rats. Next, baseline PN and tSN

activities were monitored for 40 min to evaluate any effects of adrenomedullin in both control and CIH groups. Following this period, a second bilateral microinjection of adrenomedullin was delivered into the RVLM, followed by five sequential intra-arterial injections of KCN given every 5 min, to episodically stimulate peripheral chemoreceptors. After the last injection of KCN, the PN and tSN activities were examined for up to 40 min to examine any persistent changes in these parameters induced by the combination of adrenomedullin in the RVLM and repetitive peripheral chemoreceptor stimulation. A separate group of CIH *in situ* preparations were submitted to the same experimental protocol, except that they received bilateral microinjections of saline in the RVLM.

In other groups of control and CIH *in situ* preparations, we assessed the contribution of adrenomedullin-related mechanisms to the maintenance of sympathetic activity in control and CIH groups. Baseline and KCN-induced PN and tSN activities were initially evaluated as aforementioned and then bilateral microinjection of adrenomedullin receptor antagonist (AM 22-52) were performed in the RVLM. Next, baseline tSN and PN activities of control and CIH preparations were measured at 10, 30 and 60 min after microinjections in the RVLM. Immediately after each baseline measurement, KCN injections were performed to verify the tSN and PN responses to carotid body chemoreceptor stimulation following AM 22-52 microinjections in the RVLM.

Histological processing

At the end of the study, brainstems were gently removed and placed in fixative solution (4% w/v paraformaldehyde) for 3 days, followed by cryoprotection in 20% sucrose for an additional 2-3 days. Coronal sections of 50 μm were performed to histologically confirm the sites of injection into RVLM (Leica DM IRB, Weztlar, Germany) either by the identification of the pipette track in the brain tissue or by verification of fluorescent latex microbeads (Lumafluor, New City, NY, USA) that were contained in the L-glutamate solution (1% w/v).

Data analyses

All data were digitized (5 kHz sample rate) using a CED 1401 A–D interface (CED, Cambridge Electronic Design, Cambridge, UK) and a computer running Spike 2 software (version 7, CED) with custom-written scripts for data acquisition and off-line analyses. Data

analyses were performed on rectified and smoothed signals. PN activity was analyzed by its burst amplitude (μV) and frequency (calculated by the time interval between consecutive bursts and expressed as bursts per minute, bpm). Average sympathetic activity (μV) was determined as the mean values over 1-3 min epochs. Moreover, to evaluate possible changes in the sympathetic activity due to alterations in respiratory activity, especially in the CIH rats that exhibited strengthened respiratory-sympathetic coupling (Zoccal *et al.*, 2008; Molkov *et al.*, 2011), we also analysed the average values of baseline tSN activity (μV) during the different respiratory phases (using the PN activity as a reference): inspiration (INSP, coincident with PN bursts), expiratory stage 1 (E1) or post-inspiration (the first 2/3 of expiratory phase) and expiratory stage 2 (E2) (remaining 1/3 of expiratory phase). Alterations in PN and tSN activities induced by either adrenomedullin or by AM 22-52 in the RVLM of control and CIH rats were expressed in raw units and as percentage change relative to baseline (before microinjections). During peripheral chemoreflex stimulation, changes in PN frequency was expressed as the difference between peak response and respective baseline. The tSN response to KCN was determined by an increase in the area under the curve of the integrated signal, and the changes were expressed as percentage difference from respective baseline.

The results were expressed as mean ± standard deviation of mean (SD) and were compared using unpaired Student *t*-test or two-way ANOVA with repeated measures, followed by Tukey or Dunnett *post-hoc* test, depending on the experimental protocol. The confidence level (confidence interval, CI) was set as 95% and the differences were considered statistically significant when $P<0.05$. Graphic operations and statistical analyses were performed using GraphPad Prism software (version 8, GraphPad, La Jolla, USA).

RESULTS

Sympathetic nerve activity characteristics after CIH

In situ preparations of CIH rats exhibited elevated baseline sympathetic outflows associated with enhanced respiratory-related modulation of sympathetic activity (Figure 1A) as described previously (Zoccal *et al.*, 2008). In both control and CIH *in situ* preparations (n=6/each), tSN activity displayed an increasing activity during the INSP phase reaching a peak during late-INSP or beginning of E1 (Figure 1A). In the CIH group, the levels of tSN during INSP (120.7±55.1 vs control 64.7±10.9 μV, Figure 1B, P=0.0029) and E2 phases (80.2±22.0 vs control 50.8±6.7 μV, Figure 1B, P=0.0390) were significantly higher

compared to the control group as found before (Zoccal *et al.*, 2008). No statistical differences between rat groups were noted for both the tSN during E1 phase (76.3±22.2 vs control 52.5±5.8 μV, Figure 1B, P=0.3903) and baseline PN burst frequency (22±8 vs control 18±5 bpm, P>0.9999).

Adrenomedullin in RVLM causes minor changes on baseline sympathetic activity of in situ preparations of CIH rats

Acutely, either unilateral or bilateral RVLM microinjections of adrenomedullin promoted inconsistent changes in the PN and tSN activities recorded from *in situ* preparations of both rat groups. In the control group (n=6), bilateral adrenomedullin microinjections into the RVLM were ineffective in promoting long-term changes in baseline tSN activity (Figure 2). At 20 and 40 min after microinjections of adrenomedullin, the levels of mean tSN activity (6.9±7.6 and 5.5±11 % from baseline; Figure 3A), as well as the average changes in tSN during INSP (-1.6±12.5 and -7.2±21.0 % from baseline), E1 (12.9±8.2 and 12.9±21.6 % from baseline) and E2 phases (9.7±10.3 and 10.9±21.1 % relative to baseline) were not different from baseline (P>0.05; Figure 3B). In *in situ* preparations of CIH group (n=6), bilateral adrenomedullin microinjections in the RVLM caused a transient increase in tSN activity during E2 phase at 20 min (43.9±74.4 % from baseline, P=0.05; Figure 3C), but not in the other respiratory phases (INSP: 8.0±7.3 and E1: 28.3±23.9 % from baseline, P>0.05; Figure 3C). This adrenomedullin-induced increase in sympathetic activity during E2 phase was not enough to significantly elevate mean tSN levels (26.7±17.9 % from baseline, P=0.070; Figure 3A). Forty minutes after microinjections, mean (17.4±13.0 % from baseline, Figure 3A) and respiratory-related tSN activity (INSP: 2.4±21.4, E1: 26.0±44.6 and E2: 23.9±28.4 % from baseline; Figure 3C) of CIH rats were similar to baseline levels (P>0.05). CIH animals that received microinjections of equivalent volumes of vehicle (saline) in the RVLM (n=5) did not exhibit significant changes (P>0.05) in the levels of mean tSN (4.0±5.8 and 7.4±6.1 % from baseline, respectively 20 and 40 min after microinjections; Figure 3A) and in tSN activity during INSP and E1 phases (INSP: 0.2±3.6 and 2.9±5.9 % from baseline, E1: 0.9±2.1 and 4.9±3.8 % from baseline, respectively 20 and 40 min after injections; Figure 3D), although a modest increase was noted during E2 phase (10.8±10.4 and 14.4±15.6 % from baseline, respectively 20 and 40 min after injections, P<0.0428; Figure 3D).

With respect to PN activity, microinjections of adrenomedullin in the RVLM of control group did not cause significant changes in burst amplitude (-6.7±4.8 and -6.0±6.3 % from baseline, respectively 20 and 40 min after microinjections, P>0.9999; Figure 4A), but increased burst frequency after 20 (25±11 bpm, P=0.0601; Figure 4B) and 40 min (30±14 bpm, P <0.001; Figure 4B). In the CIH group, no significant changes were noted either in the amplitude (-16.0±18.1 and -4.4±41.8 % from baseline, respectively 20 and 40 min after microinjections, P>0.999; Figure 4A) or the frequency of PN bursts (25±8 and 26±8 bpm, respectively 20 and 40 min after microinjections, P>0.05) after adrenomedullin microinjections. In the group of CIH rats that received vehicle in the RVLM, PN burst amplitude did not change (-6.0±16.7 and -11.7±16.5 % from baseline, respectively 20 and 40 min after microinjections, P>0.9999; Figure 4A), whilst frequency increased at 20 (32±5 bpm, P=0.0027) and 40 min (34±5 bpm, P=0.0006) after microinjections (Figure 4B).

Peripheral chemoreflex stimulation potentiates the facilitation of sympathetic activity induced by RVLM adrenomedullin in rats exposed to CIH

When bilateral adrenomedullin in the RVLM was combined with episodic activation of peripheral chemoreflex, a long-term sympathoexcitatory effect was observed most pronouncedly in CIH versus control groups (Figure 2). We found that mean tSN of control *in situ* preparations (n=6) significantly increased at 20 (37.9±10.9 % from baseline, P<0.0001; Figure 3A) and 40 min (47.0±15.5 % from baseline, P<0.0001; Figure 3A) after microinjections plus peripheral chemoreceptors stimulation. This sympathoexcitatory response was associated with increases in tSN mostly during the expiratory phases (E1: 46.2±24.6 and 57.3±37.6 % from baseline; E2: 42.1±16.9 and 54.7±25.1 % from baseline, respectively 20 and 40 min after microinjections, P<0.05; Figure 3B), although a significant but smaller increase was also noted during inspiration (INSP: 25.4±13.3 and 29.2±20.8 % from baseline, respectively 20 and 40 min after microinjections, P<0.05; Figure 3B). The variation of tSN caused by adrenomedullin plus carotid body chemoreceptor stimulation was higher during expiration than during inspiration (P < 0.0001).

In the CIH group (n=6), the evoked SNA response induced by adrenomedullin in the RVLM combined with episodic peripheral chemoreflex stimulation was substantially magnified relative to response observed in control rats (Figure 2). Already at 20 min after microinjections and peripheral chemoreflex stimulations, CIH rats exhibited a significant increase in mean tSN activity (61.2±30.4 % from baseline, P=0.0079; Figure 3A) that was

greater than the response observed in control rats (P=0.0491). At 40 min, a further time-related elevation was noted in the mean tSN activity of CIH rats (149.1±36.5 % from baseline, P<0.0001; Figure 3A), which was larger than in controls (P<0.0001). This progressive sympathoexcitatory response of CIH rats was initially (at 20 min) dependent on increases in tSN mostly during expiration (E1: 68.7±55.4 and E2: 87.2±109.4 % from baseline, P<0.0001; INSP: 27.7±24.3 % from baseline, P=0.3987; Figure 3C), but later (at 40 min) due to amplification of tSN activity during all respiratory phases (INSP: 108.0±82.3, E1: 161.5±69.5 and E2: 177.9±95.2 % from baseline, P<0.0001, Figure 3C). In CIH rats that received microinjections of vehicle in the RVLM combined with episodic activation of peripheral chemoreflex (n=5), we found a modest increase in mean (17.0±6.5 and 21.8±8.3 % from baseline, respectively 20 and 40 min after microinjections, P<0.001; Figure 3A) and in respiratory-related tSN activity (INSP: 16.4±15.3 and 20.2±20.9, E1: 23.8±15.5 and 30.8±19.0, E2: 10.8±10.5 and 14.4±15.7 % from baseline, respectively 20 and 40 min after microinjections, P<0.05; Figure 3D). However, the tSN response observed in CIH rats that received vehicle in the RVLM were significantly smaller than the responses observed in the other groups (P<0.05; Figure 3A).

Regarding PN activity, adrenomedullin in the RVLM in association with episodic stimulation of peripheral chemoreceptors did not alter burst amplitude of all respiratory groups (Figure 4A). In the CIH group, we verified that PN burst frequency increased after experimental procedures (30±6 and 32±6 bpm, respectively 20 and 40 min after microinjections, P<0.0036; Figure 4B), whilst no further increase were observed in the frequency of the other groups, compared to the values observed before the second round of microinjections (Figure 4B).

Acute peripheral chemoreflex responses after RVLM adrenomedullin in rats exposed to CIH

Intra-arterial KCN injections promoted prompt reflex increases in tSN activity and elevated PN frequency in control and CIH groups (Figure 5A). Before any manipulation in the RVLM, the magnitude of sympathoexcitatory response to KCN was greater in CIH than in control group (ΔtSN: 105.6±29.5 vs control 79.9±24.7 %, P=0.0480; Figure 5B), whilst the tachypnoeic response was equivalent among groups (ΔPN: 31±11 vs control 25±11 bpm, P=0.2619; Figure 5D). Bilateral adrenomedullin microinjections in the RVLM did not modify the magnitude of the sympathoexcitatory responses in either control or CIH groups (Figure

5C). On the other hand, we noticed a consistent increase in the amplitude of tachyponeic response of CIH group (P<0.01, Figure 5D), but not in controls (P>0.05). No significant changes were noted in the chemoreflex-induced responses in the CIH group that received microinjections of vehicle in the RVLM (P>0.05, Figures 5C and E).

Adrenomedullin receptor antagonism in the RVLM reduces baseline sympathetic activity of rats exposed to CIH

Microinjections of adrenomedullin receptor antagonist (AM 22-52) in the RVLM of control *in situ* preparations (n=8) did not alter mean tSN activity (66.8±24.6 vs 60.1±20.1 μV, respectively before and 60 min after microinjections, P =0.3170; Figures 6A and B). We found variable changes in tSN during the respiratory phases (INSP: 93.4±11.4, 90.0±15.7 and 89.1±18.6 % from baseline, P>0.05; E2: 101.1±13.0, 99.0±14.6 and 95.6±13.9 % from baseline, P>0.05; E1: 103.5±12.0, 102.5±33.9 and 100.5±37.8 % from baseline, P>0.999; respectively 10, 30 and 60 min after microinjections, Figures 6C-D) that were associated with a reduction in PN burst amplitude (88.0±10.2, 80.5±10.2 and 79.0±10.2 % from baseline, respectively 10, 30 and 60 min after microinjections, P < 0.0060; Figure 7A) and an increase in PN burst frequency (16±2 vs 23±9, 26±12 and 29±14 bpm, respectively before and 10, 30 and 60 min after microinjections, P < 0.0015; Figure 7B). In the group of CIH preparations (n=7), AM 22-52 microinjections in the RVLM promoted a significant reduction in basal levels of mean tSN (83.8±23.1 vs 63.9±21.7 μV, P = 0.0024; Figures 6A and B). This sympathoinhibitory response to AM 22-52 in the CIH group was associated with reductions in tSN during INSP (91.0±8.4, 81.0±16.9 and 73.7±20.4 % from baseline, respectively 10, 30 and 60 min after microinjections, P < 0.0017; Figure 6C), E2 (91.1±9.9, 80.4±12.9 and 77.7±10.7 % from baseline, P < 0.0004; respectively 10, 30 and 60 min after microinjections, Figure 6D) and during E1 (96.1±13.7, 86.3±11.7 and 79.6±12.6 % from baseline, respectively 10, 30 and 60 min after microinjections, P < 0.0412; Figure 6E). Similar to control group, a significant reduction in PN burst amplitude (94.9±8.6, 87.1±12.9 and 84.6±14.4 % from baseline, respectively 10, 30 and 60 min after microinjections, P < 0.0059; Figure 7A) and an elevation in PN burst frequency (17±5 vs 21±6, 24±7 and 28±8 bpm, respectively before and 10, 30 and 60 min after microinjections, P < 0.0437; Figure 7B) were noted in the CIH group after AM 22-52 microinjections in the RVLM.

With respect to responses to peripheral chemoreceptor stimulation, microinjections of AM 22-52 in the RVLM did not modify the magnitude of sympathoexcitatory and tachypnoeic responses of control (ΔtSN: 19.2±26.6, 32.1±39.6 and 58±66.0 % from baseline response, P> 0.05; ΔPN: 29±9 vs 27±10, 25±7 and 25±6 bpm, P>0.9999; respectively 10, 30 and 60 min after microinjections) and CIH groups (ΔtSN: 4.0±33.8; 9.6±37.8 and 15.7±42.5 % from baseline response, P>0.05; ΔPN: 33±6 vs 28±9, 29±7 and 28±7 bpm, P>0.05; respectively 10, 30 and 60 min after microinjections).

Histological verification of RVLM microinjection sites

All effective microinjections were confirmed histologically to be within the RVLM, between -12.12 and -12.48 mm relative to Bregma (according to Paxinos & Watson, 2007), and occurred at the same area where a prior microinjection of glutamate evoked sympathoexcitation (Figure 8).

DISCUSSION

The major findings of this study are: i) microinjections of adrenomedullin in the RVLM of control *in situ* rat preparations produced a sustained increase in sympathetic activity when combined with episodic stimulation of peripheral chemoreceptors; ii) the sympathoexcitatory effect of exogenously applied adrenomedullin within the RVLM associated with peripheral chemoreceptor stimulation was boosted in rats pre-treated with CIH; iii) the antagonism of adrenomedullin receptors in the RVLM reduced significantly baseline levels of sympathetic activity only in CIH rats. In contrast, we have no evidence of long-term facilitation of respiratory activity using these protocols or its modulation by adrenomedullin in RVLM.

Previous studies by Krukoff and colleagues identified pre-pro-adrenomedullin mRNA in areas of the rat central nervous system controlling hypothalamo- neurohypophyseal and pituitary axis as well as the RVLM (Shan & Krukoff, 2001a). Subsequent studies by the same group reported that adrenomedullin given either intra-cerebroventricularly in conscious Sprague-Dawley rats (Shan & Krukoff, 2001b) or microinjected into the RVLM of urethane anesthetized Sprague-Dawley rats (Xu & Krukoff, 2004) increased arterial blood pressure and heart rate; the latter effects involved glutamate acting on both NMDA and non-NMDA

receptors, and release of nitric oxide. Adrenomedullin in the RVLM was also found to inhibit the baroreflex, an effect mediated by activation of cAMP-dependent protein kinase A pathway (Xu & Krukoff, 2004, 2006). The finding that adrenomedullin in the RVLM increased blood pressure in normotensive anesthetized Sprague-Dawley rats (Xu & Krukoff, 2004) is in stark contrast to the present finding that such a manipulation (using similar concentrations of adrenomedullin) failed to change sympathetic activity in normotensive Wistar rats studied *in situ*. This may be explained by differences in the rat strains used and/or preparation employed: the *in situ* rat is decerebrate and unanesthetized and without a hypothalamus in contrast to anaesthetised, intact neuraxis *in vivo*. Alternatively, it may relate to a difference in the levels of adrenomedullin within the RVLM between the preparations used, and this may link to the presence/absence of the paraventricular nucleus [a region with adrenomedullin containing neurones (Shan & Krukoff, 2001a)], level of brainstem perfusion [higher *in situ* than *in vivo* (Paton, 1996)] and/or the level of oxygenation of the RVLM, as described below. However, this all remains to be determined.

Although adrenomedullin in the RVLM did not change baseline sympathetic outflow, the combination of exogenous adrenomedullin with episodic stimulation of peripheral chemoreceptor was able promote a persistent increase in mean sympathetic activity of control *in situ* preparations. Interestingly, this sympathoexcitatory response to adrenomedullin plus peripheral chemoreceptor activation was associated with an increase of sympathetic activity mostly during the expiratory phase. Under resting conditions, sympathetic activity that controls vascular resistance shows incrementing respiratory-related bursts during inspiration (Malpas, 1998; Zoccal *et al.*, 2009b). During hypoxia, activation of carotid body chemoreceptors promotes an increase in sympathetic activity predominantly during the expiratory phase, modifying burst pattern (Dick *et al.*, 2004; Moraes *et al.*, 2014; Machado *et al.*, 2017). Adrenomedullin microinjections in the RVLM did not potentiate the gain of sympathoexcitatory and tachypnoeic responses to chemoreceptor stimulation with KCN (at least in the time window we have explored). Based upon that, we suggest that adrenomedullin, at concentrations that does not cause acute effects, associated with the excitatory inputs triggered by peripheral chemoreceptor inputs, can promote long-lasting changes in the excitability of RVLM neurons or modify the strength of synapses on RVLM neurons, causing a sustained increase in sympathetic outflow. If this effect of adrenomedullin in the RVLM is selective to chemoreceptor inputs or can be engaged by other excitatory inputs in the RVLM, is a matter for additional studies.

In preparations of CIH rats, adrenomedullin microinjection in the RVLM did not cause significant changes in baseline sympathetic activity. On the other hand, the association of adrenomedullin with episodic peripheral chemoreceptor stimulation caused a substantial time-dependent elevation of sympathetic activity, that was not seen in CIH preparations that received vehicle in the RVLM. The adrenomedullin plus chemoreceptor activation-dependent elevation in sympathetic activity of CIH rats was associated with amplification of sympathetic activity mostly during expiration. Importantly, the antagonism of adrenomedullin receptors in the RVLM significantly reduced sympathetic activity of CIH rats primarily during inspiration and E2 phase – respiratory periods when sympathetic activity was elevated under resting conditions. The decrease in sympathetic activity promoted by AM 22-52 in the RVLM of CIH rats was associated with reductions in the respiratory drive (decrease in PN burst amplitude). The sympathoinhibitory effect of adrenomedullin receptor antagonism in the RVLM was not observed in rats that were not exposed to CIH, at least in the concentration tested (only a tendency of decrease during inspiration was observed, which might be related to reduction in inspiratory drive). Together, these findings indicate that CIH exposure sensitizes adrenomedullin-dependent mechanisms in the RVLM neurons (e.g., increased number of adrenomedullin receptors, or intracellular signaling facilitation) that, in association to augmented chemoreceptor-mediated excitatory inputs (Braga *et al.*, 2006; present study), can produce amplified and long-lasting sympathoexcitatory effects, contributing to chronically elevate arterial pressure levels (Zoccal *et al.*, 2007; Zoccal *et al.*, 2008).

Our previous work has proposed that the brainstem of both the spontaneously hypertensive rat (Paton *et al.*, 2009; Cates *et al.*, 2011; Marina *et al.*, 2015) and humans with hypertension (Warnert *et al.*, 2016) are chronically hypoperfused and may become hypoxic if arterial pressure falls, as it can do during sleep. This is relevant because hypoxia is a major stimulus for releasing adrenomedullin (Serrano *et al.*, 2002a; Serrano *et al.*, 2002b; Ji *et al.*, 2005) and is one of the few genes that can be neuroprotective to the brain (Bernaudin *et al.*, 2002). Indeed, adrenomedullin has been associated with the induced protection of remote preconditioning thereby reducing ischemia-reperfusion injury (Dong *et al.*, 2018); the latter was dependent upon activation of hypoxia inducible factor 1α. Integrating this previous evidence with the data from the present study, we propose that during CIH exposure, the central hypoxia combined with the activation of peripheral chemoreceptors might cause the release of adrenomedullin into the RVLM causing long-term facilitation of sympathetic

activity. This process may itself constitute part of the neuroprotective mechanism to ensure preserved or elevated cerebral blood flow due to the elevated arterial blood pressure resulting from increased vasoconstrictor sympathetic activity. This neurally mediated mechanism has been termed the selfish brain hypothesis of hypertension and negates low blood oxygen within the brainstem (Paton *et al.*, 2009; Cates *et al.*, 2011; Marina *et al.*, 2015; Warnert *et al.*, 2016).

A question prompted is where might the endogenous adrenomedullin originate? Whilst we do not rule out release from neurones located within the RVLM, the hypothalamus in both rats (Ueta *et al.*, 1995; Ueta *et al.*, 1999; Shan & Krukoff, 2001a) and humans (Satoh *et al.*, 1996) has been shown to contain neurons expressing adrenomedullin including the paraventricular nucleus that is known to project to the RVLM (Cato & Toney, 2005). Whether paraventricular-RVLM projecting neurones contain adrenomedullin and if they play an endogenous role in the hypertension caused by CIH in rats remains to be established.

Although adrenomedullin in the RVLM in combination with peripheral chemoreceptor stimulation caused long-term facilitation of baseline sympathetic activity, the amplitude of sympathoexcitatory reflex response to KCN was not potentiated in both groups. In contrast, the magnitude of KCN-induced tachypnoeic reflex response (dependent on a reduction in expiratory time) was amplified in CIH rats, indicating that adrenomedullin in the RVLM enhanced the central gain of peripheral chemoreflex, affecting mainly the mechanisms that control breathing during hypoxia. This effect may involve the respiratory neurons of Bötzinger and pre-Bötzinger complexes that co-exist with pre-sympathetic neurons in the ventrolateral medulla (Moraes *et al.*, 2012). The fact that this adrenomedullin-induced respiratory effect did not impact on the sympathoexcitatory reflex response to KCN suggests that the effects of ADM on RVLM neurons might be selective to neurones that are responsible for the generation of baseline sympathetic activity rather than neurones that are responsive to reflex inputs, or to specific RVLM neuronal phenotypes (e.g. catecholaminergic vs non-catecholaminergic) that exhibit differential projection targets (Ross *et al.*, 1984a; Haselton & Guyenet, 1989; Aicher *et al.*, 1996; Li & Guyenet, 1997; Abbott *et al.*, 2012; Abbott *et al.*, 2013). These hypotheses await confirmation.

Perspectives

Previous GWAS using a multi-stage design in 200,000 individuals of European ancestry described 29 SNPs associated with systolic and diastolic blood pressure, and hypertension. One of these SNPs was the adrenomedullin gene (International Consortium for Blood

Pressure Genome-Wide Association *et al.*, 2011). Given the work of Krukoff and colleagues (see above) and the present data, it is tempting to speculate that analogous mechanisms to those described herein may occur in the human brainstem in the aetiology of neurogenic hypertension. One relevant group of human patients are those with sleep apnoea. In this condition, the apnoeas cause a cyclical activation of peripheral chemoreceptors and central hypoxia producing sustained and elevated sympathetic activity with hypertension (Somers, 1995; Narkiewicz *et al.*, 1998). It now becomes important to understand the role of adrenomedullin in the human RVLM for the generation of excessive sympathetic activity in sleep apnoea and whether this might be a putative clinical target. Further work is needed to define the receptor type involved, which is challenging: there are two forms of adrenomedullin in mammals - AM1 and AM2. AM2 is also known as intermedin. Adrenomedullin signals through a unique G-protein coupled receptor - the Calcitonin receptor-like receptor (CLR) (Poyner *et al.*, 2002). CLR functions as a calcitonin gene–related peptide (CGRP) receptor or an AM receptor depending on the expression of specific receptor activity–modifying proteins (RAMPs) of which there are three (Brain & Grant, 2004). Thus, it becomes necessary to define the presence and function of receptors and RAMPs to fully understand the potential of therapeutic intervention in diseases in which adrenomedullin may work in concert with intermittent peripheral chemoreceptor input in disease states such as sleep apnoea.

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ADDITIONAL INFORMATION

Competing interests

No competing interests.

Author Contributions

DBZ, DSA, EC, BHM and JFRP contributed to conception and design of the work. DBZ, DSA, EC, KF, MPdS, JHCS, DJAM, DM and JFRP contributed to acquisition, analysis and interpretation of data for the work. All authors contributed to draft the work and revise it critically for important intellectual content. All authors approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. Baseline sympathetic activity of CIH-treated rats. **Panel A**: Representative raw and integrated (∫) recordings of baseline thoracic sympathetic (tSN) and phrenic (PN) nerve activities of *in situ* preparations from control and CIH pre-treated rat groups. **Panel B**: Average values of tSN activity during inspiration (Insp), first stage of expiration (E1) and second stage of expiration (E2) of control (n=6) and CIH (n=6) *in situ* rat preparations. * different from control group, P<0.05.

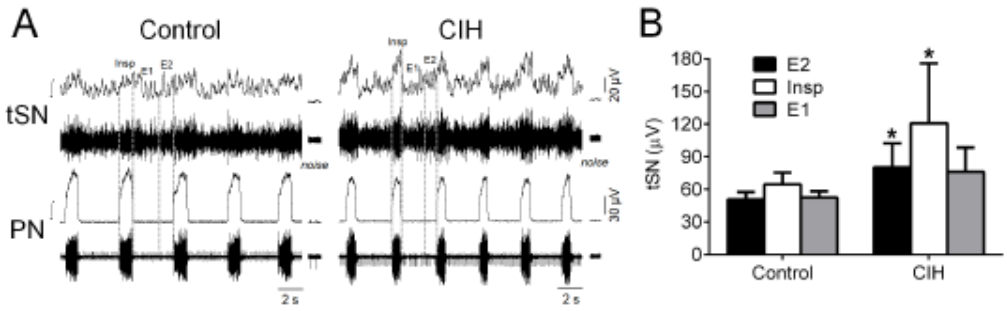

Figure 1

Figure 2. Adrenomedullin in the RVLM of control and CIH-treated rats. Representative raw and integrated (∫) recordings of thoracic sympathetic (tSN) and phrenic (PN) nerve activities of *in situ* preparations of control and CIH pre-treated rats during baseline (left), after bilateral microinjections of adrenomedullin (100 fmol/100 nl) in the RVLM (middle) and after combined bilateral microinjections of adrenomedullin in the RVLM (100 fmol/100 nl) and following episodic stimulation (5 times every 5 min) of peripheral chemoreceptors with KCN (right).

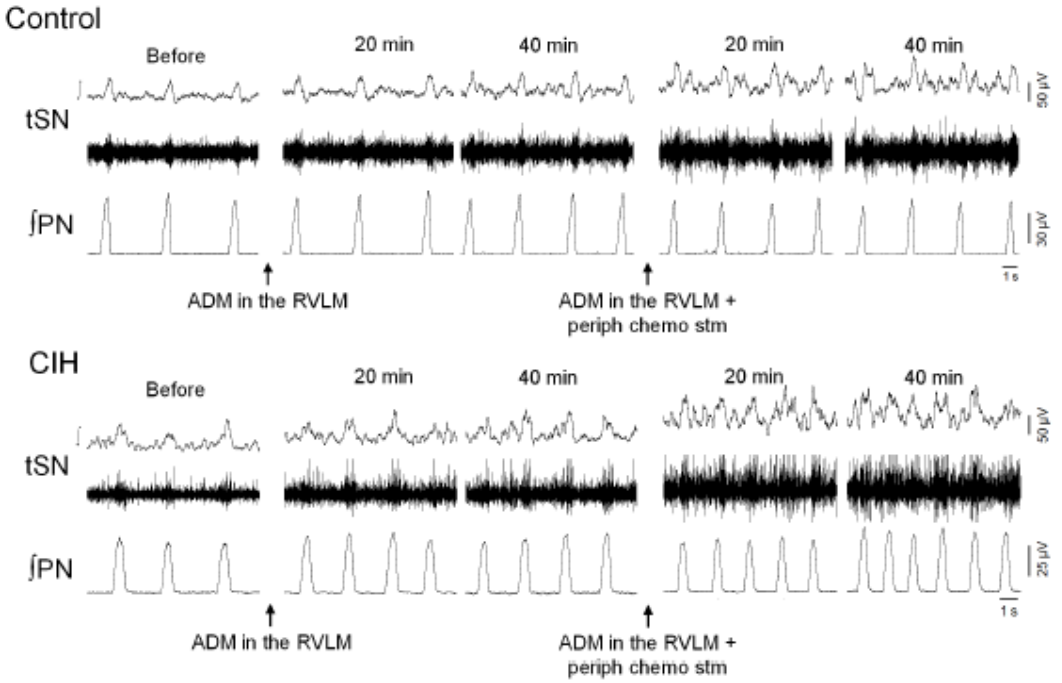

Figure 2

Figure 3. RVLM microinjections of adrenomedullin evoked increases in sympathetic activity of CIH-treated rats. **Panel A**: changes in mean thoracic sympathetic activity (tSN), relative to baseline before microinjections (100%), elicited by bilateral exogenous adrenomedullin (ADM, 100 fmol/100 nl) or vehicle in the RVLM, either alone or in combination with episodic peripheral chemoreceptor stimulation (5 times every 5 min), of control (n=6) and CIH (n=5-6) *in situ* rat preparations. * different from respective baseline; # different from control group and † different from CIH + vehicle group (P<0.05). **Panels B-D**: average changes in tSN during inspiration (Insp), first stage of expiration (E1) and second stage of expiration (E2) of control and CIH groups that received adrenomedullin microinjections in the RVLM (Panels B and C), and of CIH group that receive vehicle microinjections in the RVLM (Panel D). * different from respective baseline; # different from 20 and 40 min after adrenomedullin in the RVLM; and ϕ different from 20 min after adrenomedullin in the RVLM + episodic peripheral chemoreceptor stimulation (P<0.05).

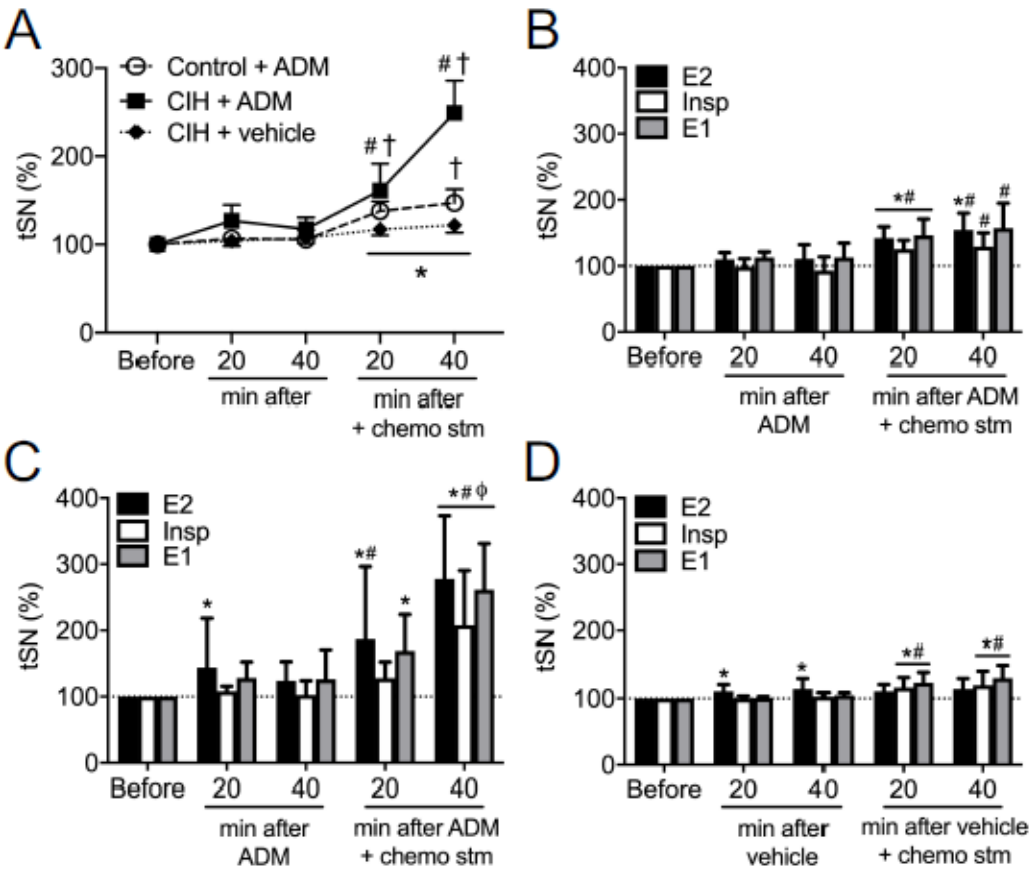

Figure 3

Figure 4. Baseline phrenic nerve activity in control and CIH-treated rats after adrenomedullin microinjections in the RVLM. Average values of baseline phrenic (PN) burst amplitude (Panel A) frequency (Panel B) of control (n=6) and CIH (n=6 and 5) *in situ* rat preparations before and after bilateral microinjections of adrenomedullin (100 fmol/100 nl) or vehicle in the RVLM, before and after repetitive stimulation (5 times every 5 min) of peripheral chemoreceptors. * different from respective baseline (P<0.05).

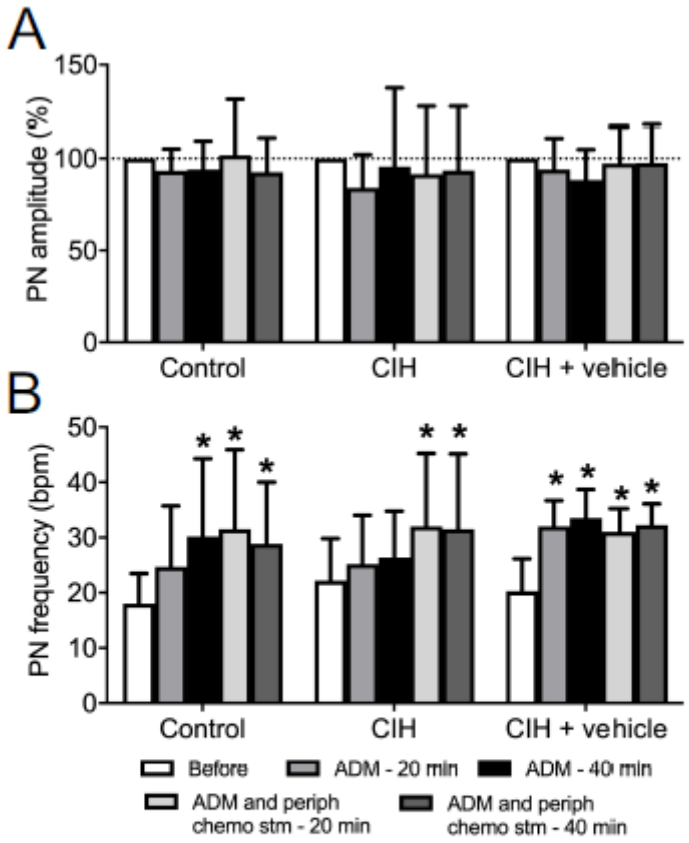

Figure 4

Figure 5. Adrenomedullin microinjections in the RVLM do not modify the acute chemoreflex sympathoexcitatory response of *in situ* rat preparations. Panel A: representative raw and integrated (∫) responses of thoracic sympathetic (tSN) and phrenic (PN) nerve activities of control (upper panels) and CIH groups (lower panels) following peripheral chemoreceptor activation with KCN (arrows) before and after (5^{th} stimulation) bilateral RVLM microinjection of adrenomedullin (100 fmol/100 nl). **Panel B**: magnitude of chemoreflex-induced increase (Δ) in the tSN activity (before microinjections) of control (n=6) and CIH-treated groups that received adrenomedullin in the RVLM. * different from respective baseline ($P<0.05$). **Panel C**: changes in the magnitude of chemoreflex-induced sympathoexcitatory response (relative to the response before microinjections in the RVLM) in control (n=6) and CIH-treated groups that received adrenomedullin, or of CIH-treated group (n=5) that received vehicle in the RVLM. **Panels D and E**: magnitude of tachypnoeic response (ΔPN) to peripheral chemoreceptor stimulation in control and CIH groups, before (Panel D) or after either adrenomedullin or vehicle injections in the RVLM (Panel E).

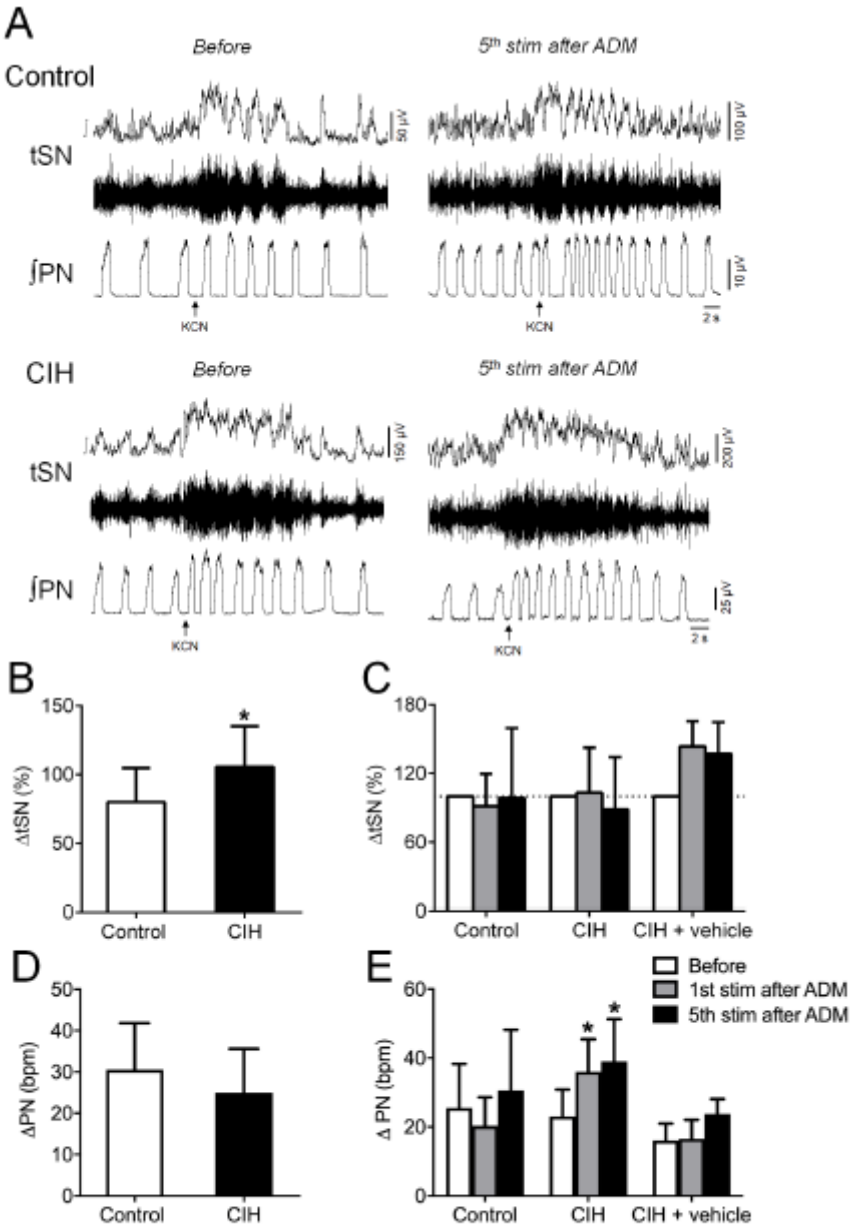

Figure 5

Figure 6. Adrenomedullin receptor antagonism in the RVLM reduces baseline sympathetic activity of *in situ* preparations of CIH rats. Panel A: raw and integrated (∫) tracings of thoracic sympathetic (tSN) and phrenic (PN) nerve activities of control (upper panels) and CIH (lower panels) *in situ* preparations, representative from their groups, illustrating the effects of bilateral microinjections of AM 22-52 (100 pmol/100 nl) in the RVLM on baseline parameters. **Panel B**: mean tSN activity of control (n=8) and CIH (n=7) *in situ* preparations before and 60 min after bilateral microinjections of AM 22-52 in the RVLM. **Panels C-E**: changes in tSN activity (relative to baseline activity before microinjections) during inspiration (Insp, Panel C), first stage of expiration (E1, Panel D) and second stage of expiration (E2, panel E) of control and CIH groups after microinjections of AM 22-52 in the RVLM. * different from respective baseline ($P<0.05$).

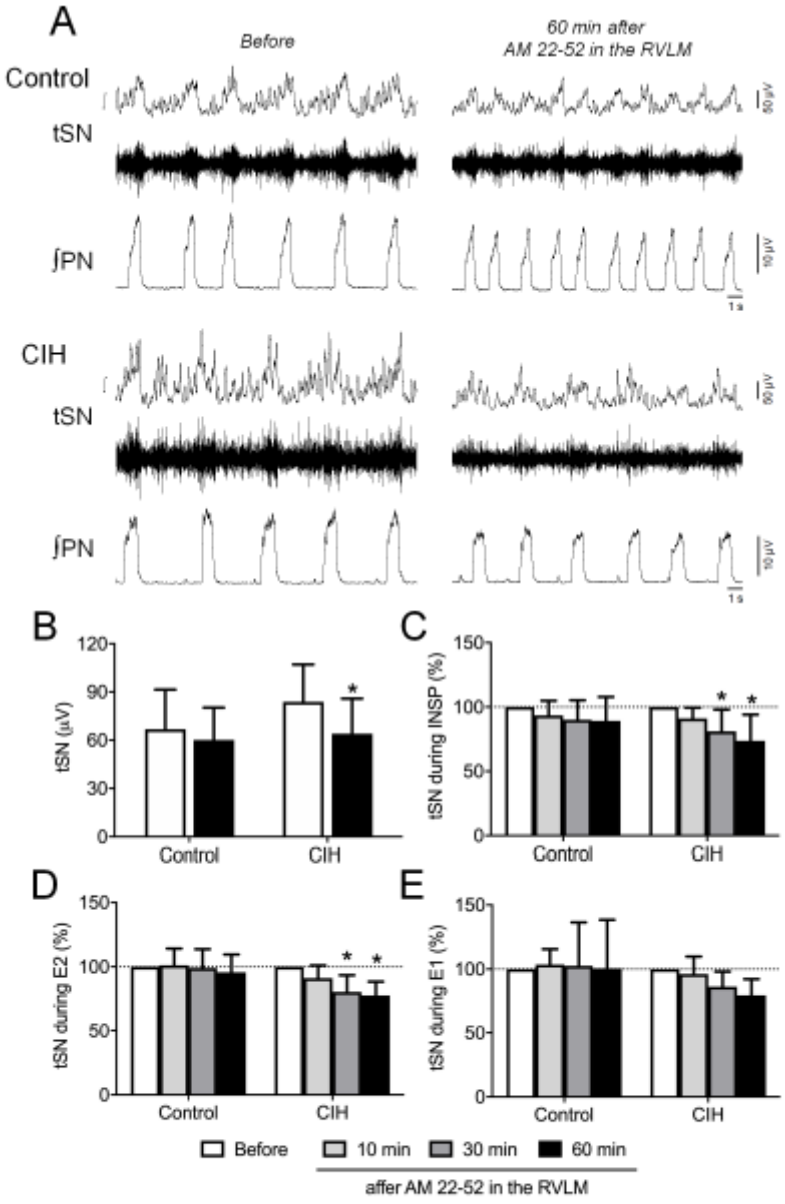

Figure 6

Figure 7. Effects of adrenomedullin receptor antagonism in the RVLM on phrenic activity of *in situ* rat preparations of control and CIH groups. Average values of baseline phrenic nerve (PN) burst amplitude (Panel A, relative to values before microinjections) and frequency (Panel B) before and after bilateral microinjections of AM 22-52 in the RVLM of control (n=8) and CIH (n=7) rat preparations. * different from respective baseline (P<0.05).

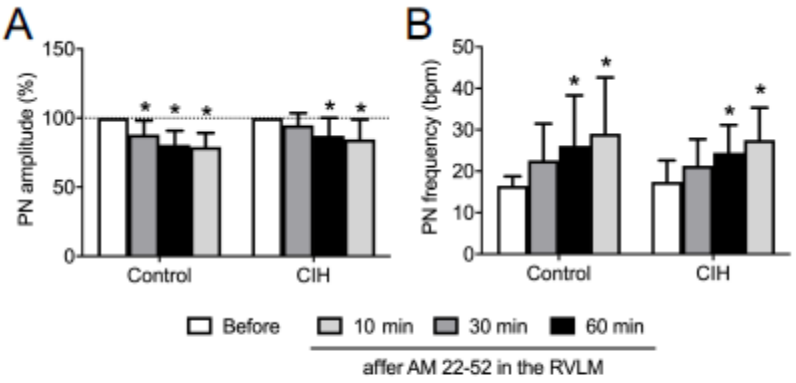

Figure 7

Figure 8. Functional and histological confirmation of adrenomedullin microinjections in the RVLM. Panel A: Raw and integrated (∫) responses of thoracic sympathetic (tSN) and phrenic (PN) nerve activities evoked by L-glutamate (1 nmol/100 nl) microinjected unilaterally in the RVLM of an *in situ* rat preparation. **Panel B**: Representative photomicrograph of a coronal section illustrating the microinjections made within the RVLM as indicated by the presence of green fluorescence beads in this region (upper panel). In the schematic drawing are indicated the approximate location of the centre of all microinjections performed in the RVLM.

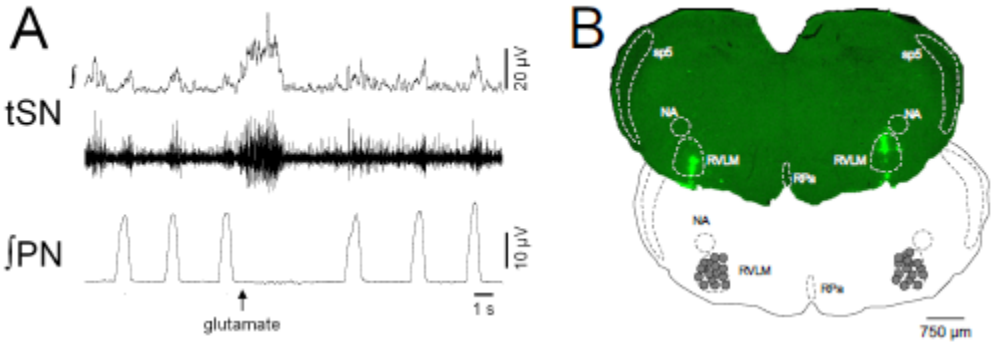

Figure 8